

# Hematopoietic cell-specific adapter proteins, SLP-76 and BLNK, effectively activate NF-AT as well as NF- $\kappa$ B by Syk and Tec PTKs in non-lymphoid cell lines

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Received 16 January 2001; accepted 6 February 2001

First published online 15 February 2001

Edited by Marco Baggiolini

**Abstract** To investigate the roles of various hematopoietic cell-specific adapter proteins in T cell receptor (TCR)-signaling leading to nuclear factor of activated T cell (NF-AT) and nuclear factor of  $\kappa$ B (NF- $\kappa$ B) activation, we reconstituted TCR-signaling with CD8/ $\zeta$ , various protein tyrosine kinases (PTKs), and adapter proteins in a non-lymphoid cell line, 293T. We show that SLP-76 and BLNK, but not LAT, effectively co-operated with Syk and Tec family PTKs to activate NF-AT and NF- $\kappa$ B. We also show that Tec family PTKs enhanced endogenous phospholipase C (PLC)- $\gamma$ 1 phosphorylation induced by CD8/ $\zeta$  and Syk in 293T cells. These results imply that PLC- $\gamma$ 1 may play a critical role in a hematopoietic cell-specific adapter protein-mediated NF-AT and NF- $\kappa$ B activation in a non-lymphoid cell. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Signal transduction; T cell receptor; Syk; Tec; Adapter; Nuclear factor of activated T cell; Nuclear factor of  $\kappa$ B

## 1. Introduction

Multiple protein tyrosine kinases (PTKs) are required to convey signals from T cell antigen receptors (TCRs) and B cell antigen receptors (BCRs). Previous biochemical studies have shown that TCR- and BCR-signaling events involve the activation of two families of PTKs; Src PTKs (Lck, Fyn and Lyn), and Syk/Zap-70 PTKs. The Src PTKs phosphorylate the two conserved tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM), and the phosphorylated ITAMs mediate the interaction of Syk/Zap-70 PTKs with the receptor, amplifying the TCR- and BCR-triggered signals by phosphorylating additional intracellular substrates including phospholipase C- $\gamma$  (PLC- $\gamma$ ), Cbl and Vav [1,2]. Tyrosine phosphorylation of PLC- $\gamma$  induces its enzy-

matic activity, leading to the activation of the inositol phospholipid pathway, followed by activation of protein kinase C (PKC) and the increase in intracellular calcium, resulting in the activation of the IL-2 promoter which is driven by different transcription factors, such as nuclear factor of activated T cells (NF-AT) and nuclear factor of  $\kappa$ B (NF- $\kappa$ B) [3,4].

TCR- and BCR-signaling require activation of Syk/Zap-70 and Src family PTKs, but requirements for other tyrosine kinases were less clear. Recently, it was found that mutations in the gene encoding a Tec family PTK, Bruton's tyrosine kinase (Btk), cause the B cell deficiency disease in humans and mice; X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice [5,6]. In vivo and in vitro studies indicate that Btk is essential for B cell survival and proliferation in response to BCR stimulation. Recently, combined deletion in mice of other two Tec PTKs, Rlk and Itk, has been shown to cause marked defects in TCR responses including cell proliferation and IL-2 production [7]. In these mice, molecular events immediately downstream from the TCR were intact, while intermediate molecular events including inositol trisphosphate production, calcium mobilization, and mitogen-activated protein kinase activation were impaired, suggesting Tec PTKs as critical regulators of TCR-signaling required for PLC- $\gamma$ 1 activation.

Most reconstitution studies with immune receptors and PTKs in non-lymphoid cells failed to activate these pathways, suggesting the requirement for additional lymphoid-specific adapter proteins [8,9]. Recently, an array of immune cell-specific adapter proteins have been identified. These adapters include linker for activation of T cell (LAT), SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), SLP-76-associated protein of 130 kDa (SLAP-130) and B cell linker protein (BLNK), which lack enzymatic and transcriptional domains, but they have multiple motifs and domains that allow binding to other proteins. It is believed that they integrate signals from surface receptors with their multiple binding sites and the potential to create various combinations of multiprotein complexes [9,10].

In the previous study, we developed a novel system to explore the TCR- and BCR-signaling pathway using a non-lymphoid cell line, 293T, which is commonly used for efficient expression of various vectors [11]. In this system, Syk, but not Zap-70, stimulated the NF-AT-mediated transcriptional response in the presence of CD8/ $\zeta$  chimeric molecules. We

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**Abbreviations:** PTK, protein tyrosine kinase; NF-AT, nuclear factor of activated T cell; PLC- $\gamma$ , phospholipase C- $\gamma$ ; NF- $\kappa$ B, nuclear factor of  $\kappa$ B

also showed that this NF-AT activation was mediated by endogenous PLC- $\gamma$ 1 and completely inhibited by inhibitors for calcineurin pathway, CsA and FK506. In this report, we demonstrate that additional expressions of lymphoid-specific adapter molecule, SLP-76 and BLNK, but not LAT, enhanced both NF-AT and NF- $\kappa$ B activation. They also enhanced endogenous PLC- $\gamma$ 1 phosphorylation induced by CD8/ $\zeta$  and Syk. Finally, we show that an additional expression of Tec family PTKs also remarkably enhanced NF-AT and NF- $\kappa$ B activation as well as enhancement of endogenous PLC- $\gamma$ 1 phosphorylation induced by CD8/ $\zeta$  and Syk. These results imply that this novel system in non-lymphoid cells may provide a useful tool to delineate or identify the regulatory molecules for the TCR- and BCR-mediated NF-AT and NF- $\kappa$ B activation.

## 2. Materials and methods

### 2.1. Cell lines and reagents

A rat basophilic leukemia cell line, RBL-2H3, was kindly donated by Dr. T. Takai (Tohoku University, Japan). A stable transformant (RBL-CD8/ $\zeta$ ) expressing CD8/ $\zeta$  was established by transfecting pTfneo-CD8/ $\zeta$  and maintained in DMEM medium supplemented with 10% fetal calf serum in the presence of 100  $\mu$ g/ml of G418. A human embryonic kidney cell line, 293T, was maintained in DMEM medium supplemented with 10% fetal calf serum. Expression vectors, CD8/ $\zeta$ , NFAT1-C, NF-AT-LUC, Zap-70, Lck, Tec, FLAG-tagged LAT, and Myc-tagged Cbl were previously described [11,12]. Expression vectors, NF- $\kappa$ B-LUC (p55Igk-LUC) [13], TecKM [14], FLAG-

tagged SLP-76, FLAG-tagged SLAP-130, Myc-tagged BLNK, Myc-tagged Cbl, FLAG-tagged LAT and Vav1 were kindly provided by Dr. T. Fujita, Dr. N. Watanabe (Tokyo Metropolitan Inst. Med. Sci., Tokyo, Japan), Dr. S. Tsukada (Osaka University, Osaka, Japan), Dr. G.A. Koretzky (University of Iowa, Iowa City, IA, USA), Dr. T. Kurosaki (Kansai Medical University, Moriguchi, Japan), Dr. A. Ullrich (Max-Planck-Inst. für Biochem., Martinsried, Germany) and Dr. H. Mano (Jichi Med. Sch., Tochigi, Japan), respectively. U73122 was purchased from Calbiochem (La Jolla, CA, USA).

### 2.2. Antibodies and flow cytometry analysis

Monoclonal anti-OKT8 was purchased from CALTAG Laboratories (Burlingame, CA, USA). Anti-Myc monoclonal antibody (mAb) 9E10, anti-CD3 $\zeta$ , anti-Syk, anti-PLC- $\gamma$ 1, anti-PLC- $\gamma$ 2, anti-Vav1, anti-Cbl and anti-SLP-76 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG M2 and anti-phosphotyrosine mAb 4G10 were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Cell staining and flow cytometry analysis were performed according to the manufacturer's instructions. Cells were stained with PE-anti-CD8 antibody (Beckton Dickinson, Mountain View, CA, USA). The stained cells were analyzed using FACS-Calibur (Beckton Dickinson, Mountain View, CA, USA).

### 2.3. Transfections and luciferase assay

10  $\mu$ g of pTfneo-CD8/ $\zeta$  vector was transfected into  $1 \times 10^7$  of RBL-2H3 cells by electroporation with single pulse conditions of 250 V and 975  $\mu$ F (Gene Pulser II, Bio-Rad). The selection and cloning for neomycin-resistant cells were performed in the presence of 100  $\mu$ g/ml geneticin (Life Technologies, Inc.). 293T cells were plated in 6-cm dishes or in 10-cm dishes and transfected by standard calcium precipitation protocol. The cells were harvested 48 h after transfection and lysed in 100  $\mu$ l of PicaGene Reporter Lysis Buffer (Toyo Ink, Tokyo, Japan) and assayed for luciferase and  $\beta$ -galactosidase activities ac-

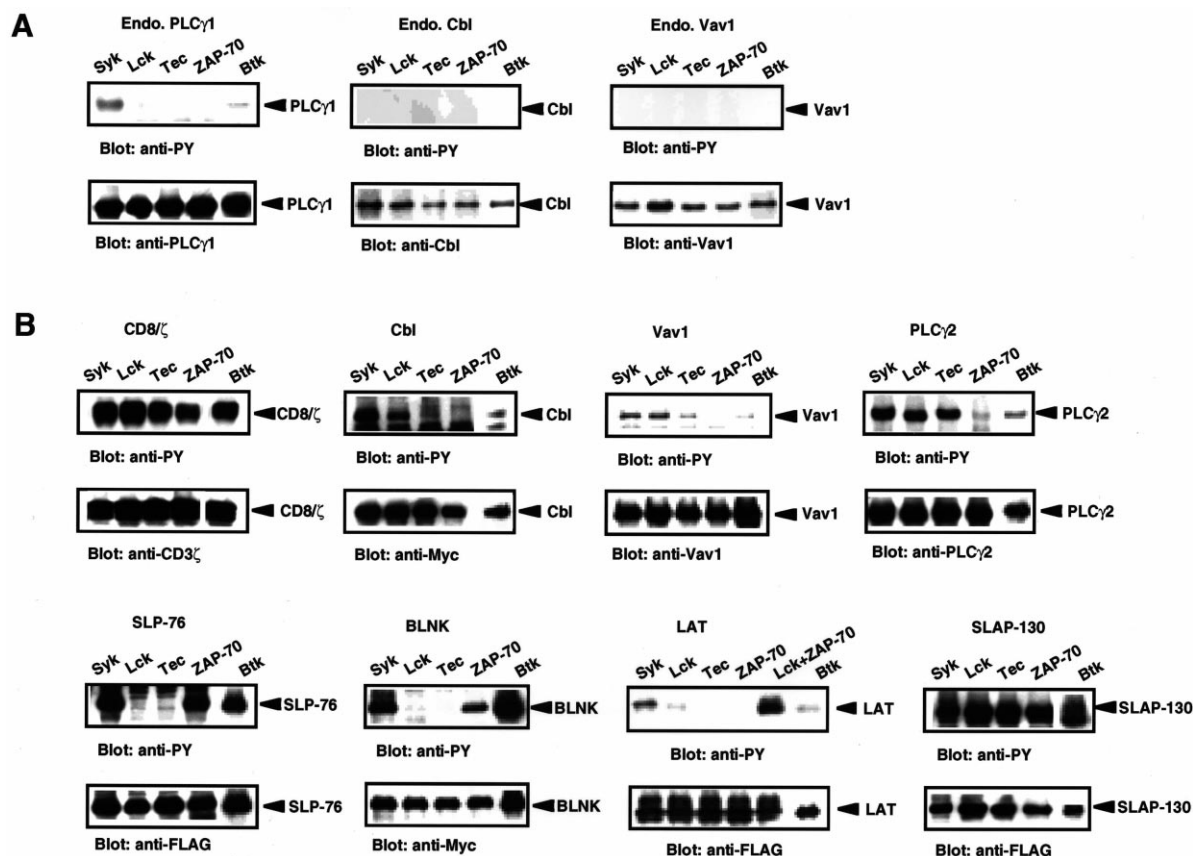


Fig. 1. Specific tyrosine phosphorylation of adapter proteins by PTKs in 293T cells. 293T cells were transfected with CD8/ $\zeta$  (5  $\mu$ g) together with each PTK; Syk (5  $\mu$ g), Lck (5  $\mu$ g), Tec (5  $\mu$ g), Zap-70 (5  $\mu$ g) or Btk (5  $\mu$ g) (A), or together with each PTK (5  $\mu$ g) and 5  $\mu$ g of Vav1, Cbl, PLC- $\gamma$ 2, SLP-76, BLNK, LAT, or SLAP-130 (B). After 48 h transfection, cells were lysed and immunoprecipitated with each antibody or anti-Tag antibody. The immunoprecipitate was probed with an anti-phosphotyrosine antibody as indicated (anti-PY, upper panels). The blot was stripped and reprobed with respective antibody (lower panels).

cording to the manufacturer's instructions. Luciferase activities were normalized to the  $\beta$ -galactosidase activities. Three or more independent experiments were carried out.

#### 2.4. Immunoprecipitation and immunoblotting

The transfected 293T cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 10  $\mu$ g/ml each of aprotinin, pepstatin and leupeptin).

The immunoprecipitates from cell lysates were resolved on 5–20% SDS-PAGE and transferred to Immobilon (Millipore, Bedford, MA, USA). The Immobilon filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Tokyo, Japan).

### 3. Results and discussion

#### 3.1. Specific tyrosine phosphorylation of adapter proteins by PTKs in 293T cells

In the previous report, we demonstrated that ectopic expression of CD8/ $\zeta$  and Syk activated NF-AT-dependent reporter gene transcription in 293T cells. To further reconstitute the CD8/ $\zeta$ - and Syk-mediated NF-AT activation in 293T cells, we first examined tyrosine phosphorylation of adapter proteins by Syk, Lck, Tec, Zap-70 and Btk in the presence of CD8/ $\zeta$ . As shown in Fig. 1A, endogenous PLC- $\gamma$ 1 was tyrosine-phosphorylated by Syk, but not Lck, Tec or Zap-70, as previously reported [11]. Faint tyrosine phosphorylation of endogenous PLC- $\gamma$ 1 was observed by expression of Btk. Nei-

ther endogenous Cbl nor Vav1 was tyrosine-phosphorylated by each PTK examined.

Next, we examined tyrosine phosphorylation of ectopically expressed adapter proteins by each PTK in 293T cells. As shown in Fig. 1B, the equal tyrosine phosphorylation of CD8/ $\zeta$  by each PTK was observed. Ectopically expressed Cbl was tyrosine-phosphorylated by Syk, Lck, and Btk, but not Tec or Zap-70. Vav1 was phosphorylated by Syk and Lck, but not Zap-70, whereas much less phosphorylation was induced by Tec and Btk. Tyrosine phosphorylation of SLP-76 and BLNK was induced by Syk, Zap-70 and Btk, but not Lck or Tec. Tyrosine phosphorylation of LAT was induced by Syk and Lck, but not Tec, Zap-70 or Btk, whereas combination of Lck and Zap-70 induced much more effective tyrosine phosphorylation than Lck alone as previously described [15]. Each PTK equally induced tyrosine phosphorylation of SLAP-130 as well as CD8/ $\zeta$ . These results suggest that Syk can tyrosine-phosphorylate all of the adapter molecules examined and is useful for reconstitution of TCR- and BCR-signaling in 293T cells.

#### 3.2. Reconstitution of NF-AT- and NF- $\kappa$ B-signaling pathway in 293T cells

To investigate whether the phosphorylation of CD8/ $\zeta$  and adapter proteins induces NF-AT and NF- $\kappa$ B activation in 293T cells, we then transfected either NF-ATc and NF-AT-LUC or NF- $\kappa$ B-LUC together with CD8/ $\zeta$  and each individual PTK and each adapter protein expression construct into

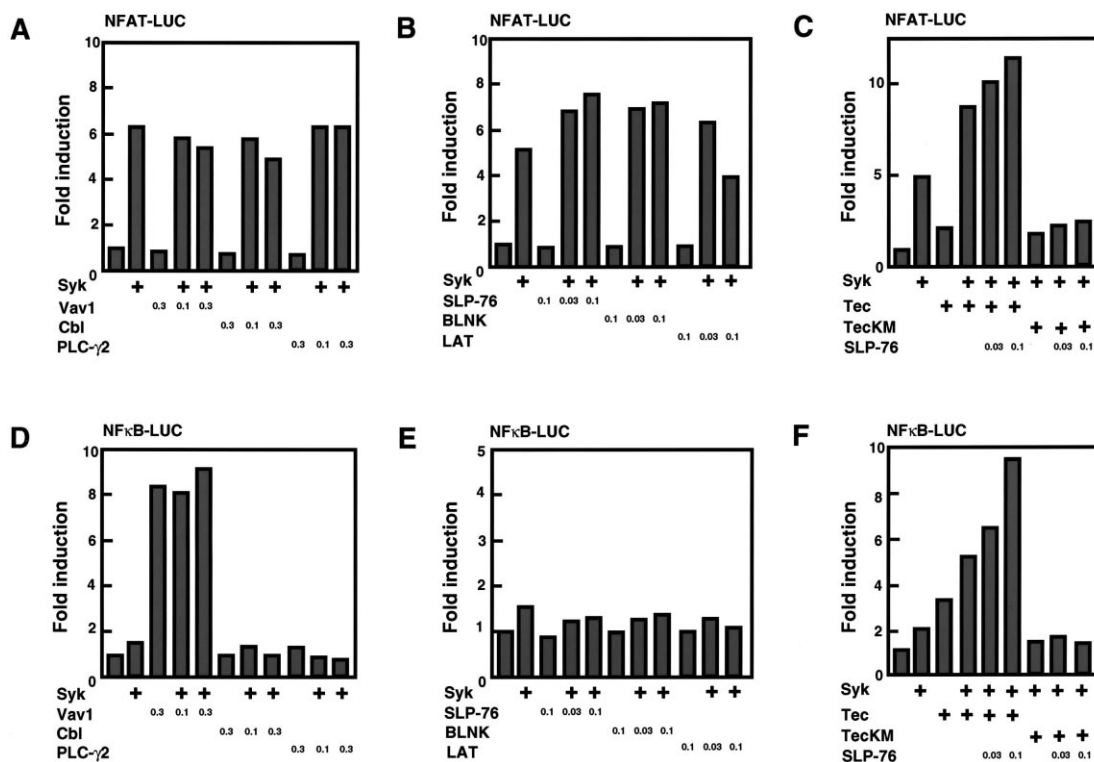


Fig. 2. Reconstitution of NF-AT- and NF- $\kappa$ B-signaling pathway in 293T cells. (A–C) NF-AT reporter assay. 293T cells were transfected with CD8/ $\zeta$  (1  $\mu$ g), NF-ATc (1  $\mu$ g), NF-AT-LUC (1  $\mu$ g),  $\beta$ -gal (0.5  $\mu$ g) with or without Syk (0.5  $\mu$ g) and increasing amounts of Vav1, Cbl or PLC- $\gamma$ 2 (A), or SLP-76, BLNK, or LAT (B), or Tec (0.5  $\mu$ g), TecKM (0.5  $\mu$ g), and an increasing amount of SLP-76 (C). (D–F) NF- $\kappa$ B reporter assay. 293T cells were transfected with CD8/ $\zeta$  (1  $\mu$ g), NF- $\kappa$ B-LUC (1  $\mu$ g),  $\beta$ -gal (0.5  $\mu$ g) with or without Syk (0.5  $\mu$ g) and increasing amounts of Vav1, Cbl or PLC- $\gamma$ 2 (D), or SLP-76, BLNK, or LAT (E), or Tec (0.5  $\mu$ g), TecKM (0.5  $\mu$ g), and an increasing amount of SLP-76 (F). 48 h after transfection, cells were harvested and luciferase activity was determined. The result shown here is representative of at least three independent experiments with similar results. S.D. was less than 10%.

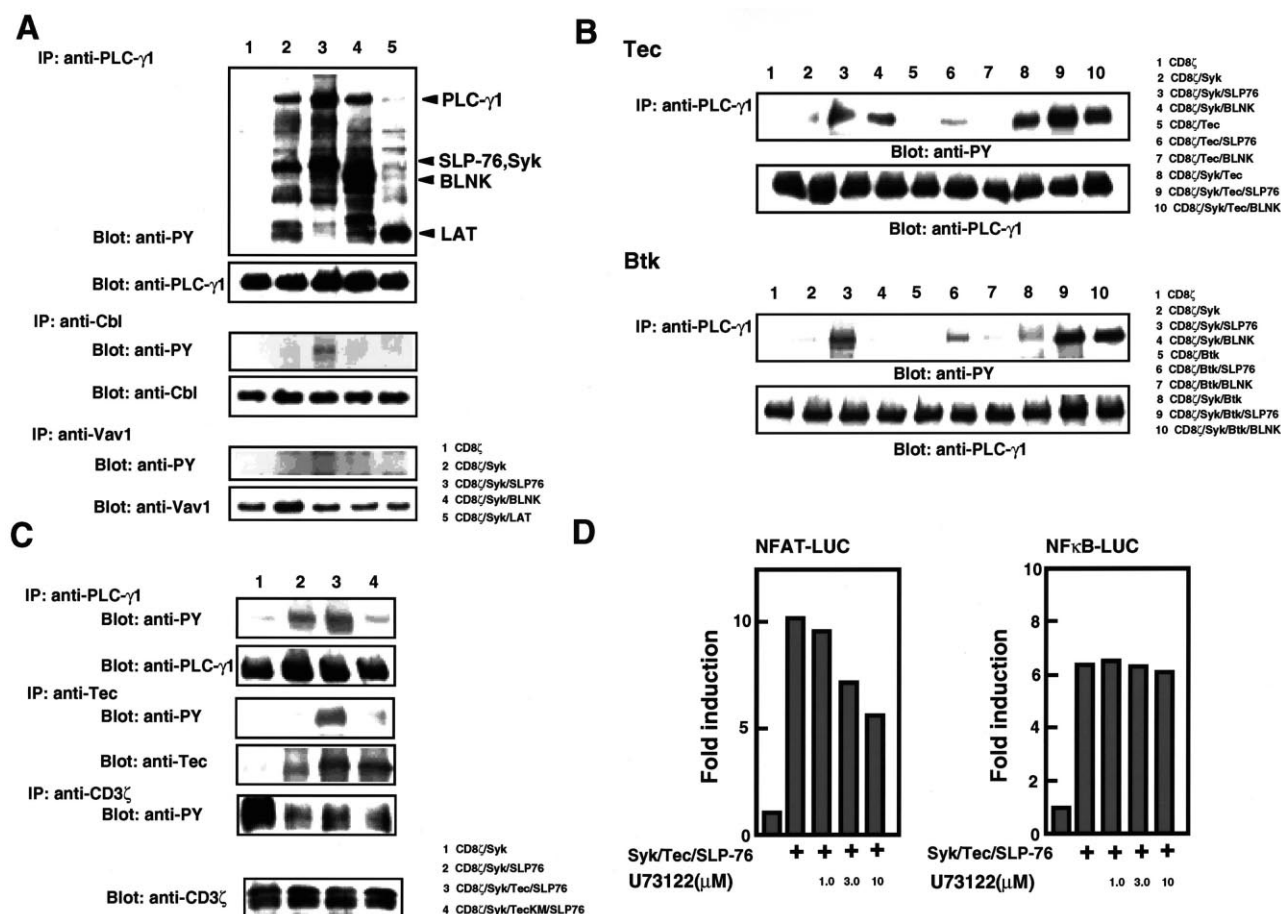


Fig. 3. Enhanced activation of endogenous PLC- $\gamma$ 1 by Syk, CD8/ $\zeta$ , Tec and adapter molecules and inhibition of NF-AT activation by a PLC inhibitor, U73122. (A–C) 293T cells were transfected with CD8/ $\zeta$  alone or CD8/ $\zeta$  (5  $\mu$ g) and Syk (5  $\mu$ g) with or without SLP-76 (5  $\mu$ g), BLNK (5  $\mu$ g) or LAT (5  $\mu$ g) (A), or with or without SLP-76 (5  $\mu$ g), BLNK (5  $\mu$ g), Tec (5  $\mu$ g), Btk (5  $\mu$ g) (B), or with or without SLP-76 (5  $\mu$ g), Tec (5  $\mu$ g), or TecKM (5  $\mu$ g) (C). After 48 h, cells were lysed and immunoprecipitated with anti-PLC- $\gamma$ 1, anti-Cbl, anti-Vav1, anti-Tec, or anti-CD3 $\zeta$ , respectively. The immunoprecipitate with each antibody was probed with an anti-phosphotyrosine antibody as indicated (anti-PY, upper panels). The blot was stripped and reprobed with respective antibody (lower panels). (D) Effect of U73122 on CD8/ $\zeta$ /Syk/Tec/SLP-76-induced NF-AT and NF- $\kappa$ B activation in 293T cells. 293T cells were transfected with CD8/ $\zeta$  (1  $\mu$ g), Syk (0.5  $\mu$ g),  $\beta$ -gal (0.5  $\mu$ g) together with NF-ATc (1  $\mu$ g), NF-AT-LUC (1  $\mu$ g) (left) or NF- $\kappa$ B-LUC (1  $\mu$ g) (right) and treated with increasing amounts of U73122 or DMSO. 48 h after transfection, cells were harvested and luciferase activity was determined. The result shown here is representative of at least three independent experiments with similar results. S.D. was less than 10%.

293T cells, and the LUC activity was assayed. As described in the previous report [11], the expression of Syk and CD8/ $\zeta$  induced a marked activation of NF-AT, whereas expression of other PTKs (Lck, Tec and Zap-70) and CD8/ $\zeta$  showed no or very little effect on the NF-AT activation. Simultaneous expression of CD8/ $\zeta$  and Syk was a prerequisite for the NF-AT activation in 293T cells. In this study, we examined effects of the additional expression of adapter proteins on not only NF-AT but also NF- $\kappa$ B activation in 293T cells. As shown in Fig. 2A,E, neither ectopically expressed Cbl nor PLC- $\gamma$ 2 augmented NF-AT and NF- $\kappa$ B activation. Significant Syk- and CD8/ $\zeta$ -dependent NF- $\kappa$ B activation was not observed in 293T cells (Fig. 2E,F). However, ectopical expression of Vav1 alone resulted in the NF- $\kappa$ B activation in 293T cells in the absence of Syk (Fig. 2E), suggesting that Vav1 is a direct activator of NF- $\kappa$ B pathway in 293T cells.

Next, we examined the effect of lymphoid-specific adapter proteins, SLP-76, BLNK LAT on NF-AT and NF- $\kappa$ B activities. As shown in Fig. 2B, an additional expression of SLP-76 or BLNK resulted in the increase of Syk- and CD8/ $\zeta$ -medi-

ated NF-AT in 293T cells coincident with their functions in lymphoid cells. Additional expression of LAT showed a decrease of NF-AT activation (Fig. 2B), although LAT is shown to be required for TCR-mediated activation of PLC- $\gamma$ 1 and the Ras pathway in T cells [16]. This finding may suggest that LAT requires additional lymphoid-specific adapter molecules for its function or other negative regulator molecules may be recruited by the phosphorylation of LAT by Syk in 293T cells.

We previously demonstrated that an additional expression of Tec markedly augmented CD8/ $\zeta$  and Syk-mediated NF-AT activation [11], supporting the previous reports showing that Btk/Tec and Syk family PTKs exert distinct effects on calcium-signaling [17,18]. Furthermore, it was shown that Btk is essential for activation of NF- $\kappa$ B via the BCR [5,6]. Thus, we examined the effect of Tec family PTKs, Tec and Btk with or without SLP-76 on NF-AT and NF- $\kappa$ B activation in 293T. As shown in Fig. 2C,F, Tec, but not TecKM (a kinase negative mutant Tec), enhanced CD8/ $\zeta$ - and Syk-mediated NF-AT activation in 293T cells. Additional expression of SLP-76 showed a slight increase of CD8/ $\zeta$ -, Syk- and Tec-mediated

NF-AT activation. Interestingly, Tec expression induced the marked increase of CD8/ $\zeta$ - and Syk-mediated NF- $\kappa$ B activation, and additional expression of SLP-76 enhanced CD8/ $\zeta$ -Syk-Tec-mediated NF- $\kappa$ B activation in 293T cells. Btk also showed a similar effect on NF-AT and NF- $\kappa$ B activation in 293T cells (data not shown). These Tec PTKs or Vav1-mediated NF- $\kappa$ B activation in 293T were completely inhibited by co-expression of a dominant negative I $\kappa$ B (data not shown). These results show that Tec PTKs, Vav1, and SLP-76 are involved in CD8/ $\zeta$ - and Syk-mediated NF- $\kappa$ B activation in 293T cells.

### 3.3. Enhanced tyrosine phosphorylation of the endogenous PLC- $\gamma$ 1 by expression of adapter proteins in 293T

In the previous report [11] as well as in Fig. 1, we demonstrated that the tyrosine phosphorylation of the endogenous PLC- $\gamma$ 1, but not Cbl and Vav1, was induced by CD8/ $\zeta$  and

Syk in 293T cells, the results indicating that endogenous PLC- $\gamma$ 1 may play a pivotal role on the NF-AT activation induced by CD8/ $\zeta$  and Syk in 293T cells. We then examined the effect of the additional expression of adapter proteins on tyrosine phosphorylation of endogenous PLC- $\gamma$ 1, Cbl and Vav1. As shown in Fig. 3A, additional expression of adapter proteins, SLP-76 and BLNK induced an increase of tyrosine phosphorylation of endogenous PLC- $\gamma$ 1 when compared with that observed with Syk and CD8/ $\zeta$ . Moreover, tyrosine phosphorylation of endogenous Cbl, but not Vav1, was induced in the presence of SLP-76, suggesting that phosphorylated SLP-76 can recruit additional adapter molecules in 293T cells. Conversely, expression of LAT resulted in a marked decrease of tyrosine phosphorylation of PLC- $\gamma$ 1 by CD8/ $\zeta$  and Syk. This finding is in accordance with the data that SLP-76 and BLNK, but not LAT, enhance CD8/ $\zeta$ - and Syk-mediated NF-AT activation in 293T cells (Fig. 2B). Furthermore, tyro-

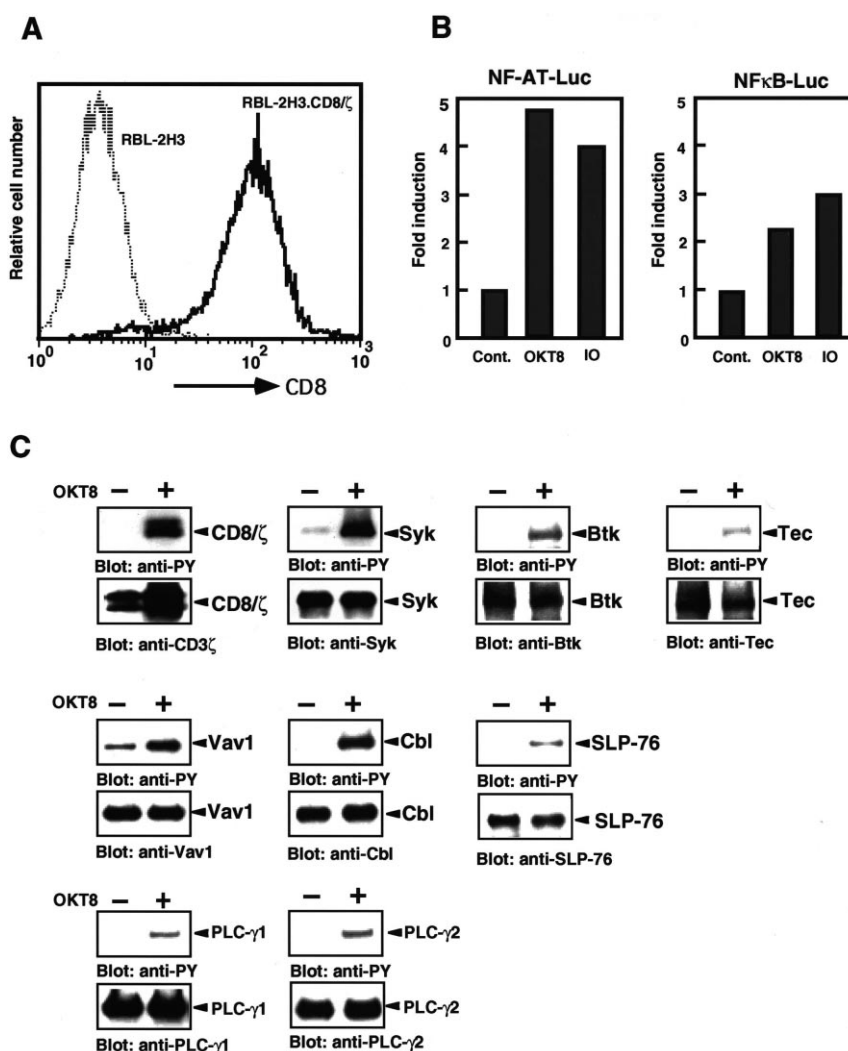


Fig. 4. The signal through CD8/ $\zeta$  induces tyrosine phosphorylation of Syk, Btk and Tec and adapter molecules in a CD8/ $\zeta$ -expressing cell line, RBL-2H3. Expression of surface CD8/ $\zeta$  on RBL-CD8/ $\zeta$  cells. RBL-2H3 (dotted line) or RBL-CD8/ $\zeta$  (solid line) cells were stained with PE-anti-CD8 and analyzed by flow cytometry. (B) NF-AT and NF- $\kappa$ B activation by OKT8 in RBL-CD8/ $\zeta$  cells.  $1 \times 10^7$  of RBL-2H3 cells were transfected with either NF-AT-LUC (15  $\mu$ g) or NF- $\kappa$ B-LUC (15  $\mu$ g) together with  $\beta$ -gal (10  $\mu$ g) by electroporation as described in Section 2. 24 h after transfection, cells were stimulated with control IgG, OKT8 antibody (5  $\mu$ g/ml) or ionomycin (1  $\mu$ M) for an additional 6 h. Cells were harvested and relative luciferase activities were measured. The result shown here is representative of at least three independent experiments with similar results. S.D. was less than 10%. (C) RBL-CD8/ $\zeta$  cells were stimulated for 15 min with OKT8 (5  $\mu$ g/ml). Cells were lysed and immunoprecipitated with each antibody. The immunoprecipitate was probed with an anti-phosphotyrosine antibody as indicated (anti-PY, upper panel). The blot was stripped and reprobed with respective antibody (lower panel).

sine phosphorylation of endogenous PLC- $\gamma$ 1 by CD8/ $\zeta$  and Syk was enhanced by additional expression of Tec or Btk in presence of SLP-76 or BLNK (Fig. 3B), suggesting that these adapter molecules recruit Tec PTKs to PLC- $\gamma$ 1 in 293T cells. This enhancement of tyrosine phosphorylation of endogenous PLC- $\gamma$ 1 by Tec was not observed by the expression of TecKM (a kinase negative mutant Tec) (Fig. 3C). Recent study demonstrated that the SH2-SH2-SH3 region of PLC- $\gamma$ 1 mediated NF- $\kappa$ B activation in a rat fibroblast cell line, 3Y1 [19]. It was also demonstrated that TNF activates PLC- $\gamma$ 2 via an upstream PTK to induce activation of PKC and PTKs, leading to NF- $\kappa$ B activation in NCI-H292 epithelial cells [20]. To examine whether activation of endogenous PLC- $\gamma$ 1 is critical in NF-AT and NF- $\kappa$ B activation in 293T cells or not, we treated the transfected cells with U73122, an inhibitor of PLC (Fig. 3D). Interestingly, U73122 had a significant inhibitory effect on NF-AT activation. On the other hand, U73122 had no effect on NF- $\kappa$ B activation in 293T cells, indicating that PLC- $\gamma$ 1 enzyme activity itself is not crucial in NF- $\kappa$ B activation as described previously [19]. The data show that activation of endogenous PLC- $\gamma$ 1 is crucial in CD8/ $\zeta$ - and Syk-mediated NF-AT activation, but not NF- $\kappa$ B activation in 293T cells.

### 3.4. The signal through CD8/ $\zeta$ induces tyrosine phosphorylation of Syk, Btk and Tec and adapter molecules in a stable CD8/ $\zeta$ -expressing cell line, RBL-2H3

To further examine the CD8/ $\zeta$ -mediated signaling pathway, we first transfected a construct of CD8/ $\zeta$  chimeric protein in a rat basophilic leukemia cell line, RBL-2H3, and obtained a stable transformant, RBL-CD8/ $\zeta$ , which showed the constitutive expression of CD8/ $\zeta$  on the cell surface (Fig. 4A). RBL-CD8/ $\zeta$  cells were transfected with NF-AT reporter luciferase construct (NF-AT-LUC) or NF- $\kappa$ B reporter luciferase construct (NF- $\kappa$ B-LUC), treated with OKT8 antibody or ionomycin, and LUC activities were determined. As shown in Fig. 4B, OKT8 as well as ionomycin stimulated NF-AT-LUC or NF- $\kappa$ B-LUC activity, suggesting that CD8/ $\zeta$  can transduce signals in RBL-2H3 cells.

We next examined tyrosine phosphorylation of cellular PTKs and adapter molecules after stimulation of RBL-2H3 cells by OKT8 (Fig. 4C). Syk, Btk, and Tec PTKs as well as CD8/ $\zeta$  were tyrosine-phosphorylated in RBL-CD8/ $\zeta$  cells. Tyrosine phosphorylation of downstream adapter molecules, such as Vav1, SLP-76, Cbl, PLC- $\gamma$ 1 and PLC- $\gamma$ 2, was also induced by stimulation of OKT8. We could not detect tyrosine phosphorylation of LAT in RBL-CD8/ $\zeta$  cells, because LAT co-migrated with highly phosphorylated CD8/ $\zeta$  molecules and co-immunoprecipitated with anti-LAT antibody (data not shown). Another lymphoid-specific adapter protein, BLNK, was not detected in RBL-CD8/ $\zeta$  cells by Western blot analysis. These results indicate that tyrosine phosphorylation of CD8/ $\zeta$  triggers the activation of PTKs and subsequent phosphorylation of adapter molecules, leading NF-AT and NF- $\kappa$ B activation in RBL-2H3 cells.

### 3.5. Conclusive remarks

The present report describes an involvement of PLC- $\gamma$ 1 in ITAM- and a hematopoietic cell-specific adapter protein-mediated NF-AT and NF- $\kappa$ B activation in the non-lymphoid cells. We demonstrated that a hematopoietic cell-specific adapter protein, SLP-76 or BLNK, but not LAT, co-operated

with CD8/ $\zeta$ , Syk and Tec PTKs in NF-AT and NF- $\kappa$ B activation in the non-lymphoid cells. In accordance with these results, tyrosine phosphorylation of endogenous PLC- $\gamma$ 1 by CD8/ $\zeta$ , Syk and Tec PTKs was markedly enhanced by additional expression of SLP-76 or BLNK but not LAT in the non-lymphoid cells. Furthermore, a PLC inhibitor suppressed this NF-AT activation but not NF- $\kappa$ B activation in non-lymphoid cells. Regarding LAT, it was shown that LAT is required for TCR-mediated activation of PLC- $\gamma$ 1 [15]. Our data imply that LAT requires additional hematopoietic cell-specific adapter molecules for its link to PLC- $\gamma$ 1 or other negative regulator molecules may be recruited by the phosphorylation of LAT by Syk in 293T cells. It has been demonstrated that overexpression of both Vav1 and SLP-76 synergistically induces TCR-stimulated NF-AT activation [21]. We found that Vav1 alone activates NF- $\kappa$ B but not NF-AT activation in 293T cells. However, no functional co-operation between Vav1 and Syk-Tec-SLP-76 was observed in 293T cells, suggesting that another adapter protein may be required for their connections on NF- $\kappa$ B or NF-AT activation in 293T cells. These results indicate that endogenous PLC- $\gamma$ 1 in 293T cells may play an important role in a hematopoietic cell-specific adapter protein-mediated NF-AT and NF- $\kappa$ B activation. Furthermore, this novel system in non-lymphoid cells may provide a useful tool to delineate or identify the regulatory molecules for TCR-mediated NF-AT and NF- $\kappa$ B activation in the downstream of CD3 $\zeta$ , Syk and Tec family PTKs.

**Acknowledgements:** We thank Dr. A. Weiss, Dr. A. Rao, Dr. T. Kurosaki, Dr. T. Saito, Dr. M. Okada, Dr. H. Mano, Dr. T. Takai, Dr. T. Fujita, Dr. N., Dr. S. Tsukada, Dr. G.A. Koretzky, Dr. T. Kurosaki, and Dr. A. Ullrich for their kind gifts of reagents. We also thank Ms. S. Hirota for her technical assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture in Japan.

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